RESEARCHREPORT
PORK SAFETY

Title: Prevalence of *Yersinia enterocolitica* in US Market Weight Hogs
NPB# 00-135

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Abstract: *Y. enterocolitica* is a major human food-borne pathogen which accounts for ~87,000 human cases and ~1,100 hospitalizations each year in the US. The purpose of this study was to estimate the individual animal and herd prevalence of *Y. enterocolitica* in hogs sampled during National Animal Health Monitoring System (NAHMS) Swine 2000. Tonsil and fecal swabs were collected on farms (n=122) located in the 17 pork producing states. Tonsil (n=1,218) and fecal (n=2,033) samples were tested by our laboratory at the National Animal Disease Center (NADC) for the *ail* gene. Results from an additional 814 fecal samples analyzed by Dr. S. Bhaduri,USDA-ARS-Eastern Regional Research Center (ERRC) were incorporated into the final analysis of fecal samples (total=2,847). The *ail* gene of *Y. enterocolitica* encodes the adherence and invasion protein which is a major virulence factor. The fluorogenic 5′ nuclease PCR assay, which was developed earlier in this laboratory with financial support of the National Pork Producers Council, was further optimized and used to screen hogs for the *ail* gene of *Y. enterocolitica*. For tonsils (122 of 1,218 samples), 10% were positive in irgasan tiracillin chlorate (ITC) enrichment broth whereas 5.6% (68 of 1,218) of samples were positive after subculture to the more selective cefsulodin-irgasan-novobiovin (CIN) agar. This indicates that growth in ITC broth is more permissive for pathogenic strains than the more selective CIN agar. Thus, data based solely on results obtained by CIN culture may under estimate the prevalence of *Y. enterocolitica*. When tonsils were sampled to monitor the hog carrier status, the herd prevalence based on ITC data is ~32% (32 of 100 premises sampled) whereas the prevalence based on growth on CIN is ~20% (20 of 102premises). When feces were used to gauge *Y. enterocolitica* prevalence, an estimated 13% of hogs harbored the *ail* gene, after

ITC enrichment (372 of 2,847). The overall herd prevalence is estimated at 45.08% (55 of 122 premises), based on the detection of the *ail* gene in ITC broth enrichments of tonsils and combined laboratory data of feces. We have correlated herd prevalence data with farm management practices. Initially, 12 factors were selected on the basis of passing the cut-off value of $p<0.25$. Next, five factors were identified as correlated with herd prevalence at the 90% probability level ($p<.10$). These included antibiotics in the feed ($p = 0.07$), previous history of vaccination for *E. coli* ($p = 0.09$), deaths reported on the premises which were due to scours ($p = 0.07$), failure of pigs to thrive because of the porcine respiratory distress complex (age at which pigs "hit the wall") ($p = 0.07$), and use of meat/bone meal in grower-finisher diet ($p = 0.10$). Ultimately, four factors were identified in the final regression model. These included, with their accompanying odds ratio (OR), location in a non-central state (OR=0.3), vaccination for *E. coli* (OR 3.0), % deaths due to scours (OR 3.5), and presence of meat/bone meal in grower-finisher diet (OR 4.1).

Introduction: *Y. enterocolitica* is a major human food-borne pathogen which causes ~87,000 human cases and ~1,100 hospitalizations each year in the US. Swine are an important animal reservoir of *Y. enterocolitica* strains, including serotypes O:3 , O:8, O:9, and O:27, which are pathogenic in humans (1, 3, 4, 5, 7, 11). In one study, virulent strains of *Y. enterocolitica* 0:3 were found in the oral cavity and the gastrointestinal tract of pigs (10). In the US, serotypes O:8 and O:5,27 predominate (3, 4, 7, 11).

Virulent strains of *Y. enterocolitica* which are pathogenic to man harbor the *ail* gene, which encodes the adherence and invasion protein (9, 10). We have developed a second generation, fluorogenic PCR (TaqMan) assay to detect the *ail* gene of *Y. enterocolitica* (1,5). The assay is more sensitive than culture or first generation PCR protocols (1). The 5´ PCR assay differs from 1st generation assays in that it utilizes a fluorogenic probe, which emits fluorescence (8). The increase in fluorogenic signal is directly related to the PCR amplification of specific DNA targets (8). A signal which exceeds the threshold indicates the presence of the *ail* gene of pathogenic *Y enterocolitica* (2, 6, 12, and Figure 2).

In hogs, *Y. enterocolitica* is spread via the fecal oral route (1, 4, 7, 11). Because swine are the major animal reservoir of strains pathogenic for humans, there is a need to monitor hogs for the virulent (*ail*-harboring) strains of *Y. enterocolitica*. Once a baseline is established, continuous monitoring may be used to assess the effectiveness of on-farm pathogen reduction programs. The NAHMS Swine 2000 study, which included a questionnaire as well as on-farm sampling, canvassed 1,000 operations from the top 17 hog producing states. This represents 92% of the US hog inventory and 75% of operations.

The goal of this study was to screen a statistically valid number of market weight hogs during the NAHMS Swine 2000 for the *ail* gene of pathogenic *Y. enterocolitica* with the TaqMan assay. We analyzed both tonsil swabs and fecal samples to better estimate the carrier status of market weight hogs. Second, based on laboratory results, statistical analysis by the USDA-APHIS CEAH staff may indicate which management practices are risk factors for infection.

Knowledge of these factors is critical in reducing *Y. enterocolitica* in the farm to table continuum.

Objectives from Research Proposal:

A. Determine the animal-level prevalence of pathogenic *Y. enterocolitica* in hogs by culture and TaqMan methods;
B. Based on resultant herd prevalence, in collaboration with USDA-APHIS-CEAH, identify on-farm management factors linked with *Y. enterocolitica* in swine.

Procedures:

Field collection: On-farm sampling by USDA-APHIS federal and state field veterinarians was conducted from August through October 2000 and in the spring of 2001. Sampling of a subset of hogs during NAHMS 2000 assured a random sampling of US swine located in the 17 pork producing states including, in alphabetical order, Arizona, Colorado, Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, North Carolina, Ohio, Oklahoma, Pennsylvania, South Dakota, Texas, and Wisconsin.. The proportion of samples collected from each state reflected that state's contribution to the overall national swine population. Assuming a hog prevalence of 5% in the Midwest (5), this sample size estimated animal level prevalence with a 95% probability. Fecal samples were actually collected from a 129 sites, 50 samples per site from 10 pens with late finishers and 25 samples from cull sows. Tonsil swabs were taken from 12 late finishing pigs per site on 115 sites. Prior to field sampling, a NAHMS training session with field veterinarians was held at USDA-APHIS-CEAH headquarters in Ft. Collins, Colorado, to instruct field veterinarians on the public health significance of *Y. enterocolitica* in hogs and to provide practical instructions on collection and shipment of tonsil swabs.

Questionnaire: A questionnaire, designed in collaboration with USDA/National Agricultural Statistics Service and modified from the NAHMS Swine '95 study, assessed general farm management practices. Producers who completed the questionnaire were invited to participate in the on-farm testing phase of the NAHMS Swine 2000.

Sample enrichment procedure: Oropharyngeal (tonsil) swabs were collected from hogs (n=1,218) using Copan swabs, placed in Amies transport media (Starswab, Starplex Scientific, Ontario, Canada), and shipped via overnight mail to the National Animal Disease Center (NADC), Ames, Iowa. Pen floor fecal samples were collected in 50 ml conical centrifuge tubes and shipped overnight to USDA-ARS laboratory in Athens, Georgia. From this central collection point, fecal samples were then distributed on alternate weeks to the Ames NADC and Eastern Regional Research Center (Dr. S. Bhaduri, ERRC) laboratories. Upon arrival at NADC, tonsil swabs were enriched in 10 ml irgasan tiracillin chlorate (ITC) media (2 days at room temperature). After enrichment 100 µl ITC was streaked to *Yersinia* selective media, cefsulodin-irgasan-novobiovin (CIN) agar (25C, 48 hrs). One typical *Yersinia* "bull's eye" colony from each CIN plate was

selected for confirmation via the 5' fluorogenic assays as described (Figures 1,2 and reference 6).

DNA extraction: DNA was prepared from all the ITC samples as follows. A 1 ml aliquot from the ITC media was centrifuged (2 min, 10,000 x g), the supernatant was carefully removed, and the remaining pellet was resuspended in 200 µl PrepMan sample preparation reagent (PE Applied Biosystems). The tubes were vortexed to resuspend the pellet and boiled (102 °C for 10 min). The tubes were centrifuged (2 min at 10,000 x g) to pellet cell debris. The supernatant was transferred to a microcentrifuge tube and stored (-20C). DNA was also extracted from the presumptive *Yersinia* colonies on the CIN plates and stored (-20 °C) until PCR analysis.

Fluorogenic 5′ nuclease PCR conditions: The probe and primer sequences, which target the *Y. enterocolitica ail* gene, are given in Table 1 (6). Oligonucleotide primers and the fluorescently labeled probe were synthesized by Integrated DNA Technologies (Coralville, Iowa). The probe was labeled at the 5′ end with fluorescent reporter dye FAM-6-carboxy-fluorescein and the 3′ end with the quencher dye TAMRA-6-carboxy-tetramethyl-rhodamine. The PCR conditions were described earlier (6). PCR reactions were performed in a 96-well format in the PE ABI PRISM™ 7700 Sequence Detection System (PE Applied Biosystems). Briefly, each PCR mixture contained 3.5 mM $MgCl_2$, 0.2 mM each of deoxynucleoside triphosphate, 1.25 U of AmpliTaq Gold DNA polymerase (PE Applied Biosystems), and 1 X GeneAmp PCR Gold Buffer (150mM Tris-HCl, 500 mM KCl) (PE Applied Biosystems). 5 µl of DNA template was added to yield a total volume of 50 µl. Concentrations of primers (200 mM) and the probe (25 nM) as originally described (6) yielded inconsistent results. Therefore, primers (50 nM to 900 nM) were tested in a matrix format with a known positive DNA template. Optimal concentrations of 300 nM for both the forward and reverse primer were then used to screen the tonsil samples. Probe concentration was also increased from 25 nM to 200 nM. The thermal cycling conditions were as follows: 95° C for 10 minutes and 35 cycles of 95° C for 15 seconds (denaturation) and 58° C for 1 minute (primer annealing and extension) followed by an indefinite hold at 25° C (6).

Analysis was completed on the ABI 7700 Sequence Detection System. Cycle 33 was set as an arbitrary cut off point. Thus, any sample that crossed the threshold prior to cycle 33 and showed logarithmic amplification was scored as positive (Figure 2). Any sample that crossed the threshold after or on cycle 33 or did not show sufficient amplification was scored as negative

Results :

Objective 1: Determine the animal-level prevalence of pathogenic *Y. enterocolitica* in hogs by culture and PCR-based methods. *ail*-bearing *Y. enterocolitica* were detected more frequently in ITC enrichments than from CIN plates. As summarized in Table 2, 10.0% of tonsil samples (122 of 1,218 samples) were positive after ITC enrichment whereas only 5.58% of samples (68 of 1,218 samples) were positive after subculture to selective CIN media. This indicates selective plating may compromise the recovery of *Yersinia* and that

data based solely on selective plating may underestimate its prevalence. Likewise, for fecal samples examined at NADC, 16.04% (362 of 2,033 samples) were positive by ITC enrichment. A lower recovery, 6.35% positive (129 of 2,033 samples), was seen when ITC enrichments were subcultured to CIN.

As summarized in Table 2, since both NADC and ERRC (Dr. S. Bhaduri) laboratories utilized a common ITC enrichment for fecal culture and the 5' TaqMan assay, data were combined to better estimate the herd prevalence. As a result, ~13% (372 of 2,847 samples) of fecal samples were positive. Overall herd prevalence was based on combining results from ITC enrichment of tonsils and feces. An estimated ~47% (55 of 122 premises) of farms yielded at least one positive sample. The individual animal prevalence (10%) based on tonsil swabs, is less than that reported earlier for hogs at slaughter in the Midwest (13.2%) (5). However, the herd prevalence of 30% achieved in this study, based on culture of tonsils in ITC, is comparable to the 28.2% of positive groups of hogs (=lots) reported for the Midwest (5). The current study sampled pigs on-farm and therefore involved no transport stress or antemortem contamination while in the holding pens immediately prior to slaughter. This study also differs from earlier reports in that as a national survey it monitored hogs from 17 states.

The highly sensitive fluorogenic PCR assay detected the *ail*-bearing strain of *Y. enterocolitica* with almost equal frequency in tonsil swabs (10. 02%, 122 of 1,218 samples) and feces (13.06%, 372 of 2,847 samples). This suggests that herculean measures, such as collecting tonsil swabs, may not be needed to gauge the prevalence of *Y. enterocolitica* in the live hog.

Objective 2: Based on resultant animal prevalence, in collaboration with USDA-APHIS-CEAH, identify on-farm management factors linked with *Y. enterocolitica* in swine.

Data management: The completed Fecal Collection Record (FCR) was received from the field and entered directly into SAS via a data entry screen. Data validation checks were run after each day of data entry using the "FecalVal.sas" program and data were stored into the fecal.sas7bdat dataset. At the conclusion of sample collection, the completed FCR dataset was validated using the "FCR.check.sas" program (October 2001) which runs 20 edit checks. Additional follow-up edit checks were also added to this program and rerun that same month. Edits were made to FCR data via the "FCR.fix.sas" program. This program was updated and finalized on November 1, 2002. It creates the fcrclean.sas7bdat dataset which has one observation for each farm.

The "NAHMS S2K Salmonella.xls" file was received from USDA-Athens, Georgia (Dr. Paula Cray). The "FCR pen list" generated a worksheet with the sample numbers by pen for each kit from the fcrclean.sas7bdat dataset. The number of isolates positive for Salmonella were recorded for each pen along with the serotypes and serogroups isolated from each pen. This was saved in the "Salmonella results.xls" spreadsheet which was imported into SAS and saved as the salmrslt.sas7bdat dataset. Some minor checks and edits were made via the "Salmonella.results.sas" program to both the salmrslt.sas7bdat data and to the

fcrclean.sas7bdat data (p#numb series which list the sample numbers for each pen). These data, along with count of samples tested for Salmonella and Campylobacter from the fecalmast.sas7bdat dataset, were merged together to create the salmonella.sas7bdat dataset. Finally, code was written to similarly insert the Salmonella results for culls sows.

The "Table 2-NAHMS-Final Summary" sheet, a print out of a xls file, was received from NADC. An overall outcome for each kit was entered using the "*Yersinia* results.sas" program. This program adds the *Yersinia* outcome for each kit to the salmonella.sas7bdat dataset and creates the yersinia.sas7bdat dataset. Then, based on discussions and review of the literature, selected variables from the questionnaires were merged to create the yersrisk.sas7bdat dataset. A basic frequency analysis of the variables was conducted and minor edits made to the dataset.

Definition of outcome: The "Table 2-NAHMS-Final Summary" spreadsheet of results contained for each kit the number of tonsils tested and positive by ITC and CIN and the number of fecal samples tested and positive by ITC. A site was classified as positive if any of these three methods identified at least one positive sample.

Yersinia results were obtained on 115 of the 129 sites participating in the fecal collection phase. Nearly 45% of the sites (55/122) were classified as positive. The prevalence of positive sites was lower in Northern and Southern tier states (33-37%). Sites sampled in the fall (Initial VMO visit) were less likely to be positive (40.7%) than those sampled in the winter (59.3%) on the Second VMO visit.

Prior to field sampling, questionnaires were sent to participating farms. The responses to the questionnaires identified 40 on-farm management factors. Twelve risk factors were initially linked with either the presence or absence of *Y. enterocolitica* in hogs ($p<0.25$). These are highlighted and summarized on Table 3. Factors which were related at the 90% probability level ($p < 0.1$) included antibiotics in the feed ($p= 0.07$), previous history of vaccination for *E. coli* ($p=0.09$) as well as deaths reported on the premises which were due to scours ($p=0.07$). The failure of pigs to thrive because of the porcine respiratory distress complex, which slows weight gain (age at which pigs “hit the wall”) was associated with *Y. enterocolitica* ($p=0.07$). Use of meat/bone meal ($p=0.10$) and use of animal/vegetable fat ($p=0.18$) in the grower-finisher diet were associated with *Y. enterocolitica*. However, use of fish meal in the grower finisher diet ($p=0.55$) was not. Access of rodents to buildings ($p=0.19$) was linked to *Y. enterocolitica* in hogs. However, using either cats or dogs as rodent control as well as presence of livestock trucks coming on site were not risk factors in this study. In Europe both the presence of cats as well as transport vehicles on-site have been related to the prevalence of *Y. enterocolitica*. We hypothesized that the presence of other enteric pathogens predisposed to yersiniosis. However, in our analysis for US hogs, presence of *Salmonella*, roundworms, gastric ulcers, and ileitis were not a significant predisposing risk. We hypothesized that since *Yersinia* is present in other livestock that there could be the opportunity for

transmission. Yet accessibility of buildings to birds, cats, wildlife as well as the presence of cattle, sheep or goats on site or feral pigs in the county were not risk factors in this study. Ultimately, four factors were identified in the final regression model. These included, with their accompanying odds ratio (OR): location in a non-central state (OR=0.3), vaccination for *E. coli* (OR 3.0), % deaths due to scours (OR 3.5), and presence of meat/bone meal in grower-finisher diet (OR 4.1).

Summary

This study confirmed that virulent species of *Y. enterocolitica* harboring the *ail* gene are present in tonsil and fecal swabs of US swine with an overall herd prevalence of 45.08% (55 of 122 premises). Improved detection methods using the 5'nuclease protocol suggest that the microbe may be found in either the tonsils (10.0%, 12 of 1,218 hogs) or feces (13.1%, 372 of 2,847 hogs). Of the 40 on-farm management practices analyzed, 12 were initially identified as probable risk factors for *Y. enterocolitica* ($p<0.25$). Ultimately, four factors were identified in the final regression model. These included, with their accompanying odds ratio (OR); location in a non-central state (OR=0.3), vaccination for *E. coli* (OR 3.0), % deaths due to scours (OR 3.5), and presence of meat/bone meal in grower-finisher diet (OR 4.1).

Portions of these studies have and will be been presented to the annual meetings of the Food Safety Consortium (Ames, Iowa, September 2001), American Society for Microbiology (Salt Lake city, Utah, May 2002), American Association of Swine Veterinarians (Kansas City, Missouri, March 2002), and have been published in the 2001 Iowa State Swine Research Report.

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TABLE 1. Annealing site and sequence of primers and probe used in the assay.

Primer/probe	Annealing site	Sequence (5′ • 3′)
TM1-F	746 to 772	GGT CAT GGT GAT GTT GAT TAC TAT TAC
TM1-R	818 to 836	CGG CCC CCA GTA ATC CAT A
TM1-P	785 to 814	CCA TCT TTC CGC ATC AAC GAA TAT CTT AGC

TABLE 3. Univariate analysis

Factor	n	Percent positive	P value	OR
Herd size			0.36	
<1500	51	45.1		
1500-3000	33	60.6		
3000+	29	48.3		
Facility type - GF			0.76	
No outside access	92	51.1		
Outside access	17	47.1		
Waste management - GF			0.82	
Non-flush system	92	50.0		
Flush system	17	52.9		
Pig flow - GF			0.31	
Continuous	31	58.1		
Not continuous	78	47.4		
Deworm regularly			0.15	
Yes	48	58.3		
No	65	44.6		
Mange/Lice regularly			0.78	
Yes	23	47.8		
No	90	51.1		
Antibiotics in feed			0.07	
Yes	94	54.3		
No	19	31.6		
Vaccination for E. coli			0.09	
Yes	41	61.0		
No	72	44.4		
Restrict farm access			0.93	
Yes	65	50.8		
No	48	50.0		
Livestock trucks come on site			0.90	
Yes	72	50.0		
No	41	51.2		
Rodent control - use cats			0.78	
Yes	51	49.0		
No	62	51.6		
Rodent control - use dogs			0.96	
Yes	28	50.0		
No	85	50.6		
Percent deaths due to Scours			0.32	
<6%	87	48.3		
>6%	18	61.1		
Percent deaths due to Scours			0.07	
0 %	81	45.7		
1-100%	24	66.7		
Percent deaths due to Resp.			0.74	
<50%	67	49.2		
50-100%	38	52.6		
Percent deaths due to Stress			0.59	
0 %	68	48.5		
1-100%	37	54.0		

Factor	n	Percent positive	P value	OR
PRRS – GF			0.45	
Yes	30	56.7		
No	78	48.7		
Salmonella – GF			0.77	
Yes	9	55.6		
No	99	50.5		
Atrophic Rhinitis – GF			0.24	
Yes	19	63.2		
No	89	48.3		
HBS – GF			0.74	
Yes	25	48.0		
No	83	51.8		
Ileitis – GF			0.70	
Yes	53	49.1		
No	55	52.7		
Gastric Ulcers – GF			0.30	
Yes	25	60.0		
No	83	48.2		
Roundworms – GF			0.48	
Yes	17	58.8		
No	91	49.5		
Age pigs hit 'the wall'			0.07	
< 12 weeks	8	25.0		
12–17 weeks	41	39.0		
18+ weeks	11	72.7		
Number of times resorted			0.61	
0	32	43.7		
1	49	53.1		
2+	27	55.6		
Fish meal in GF diet			0.55	
Yes	10	60.0		
No	98	50.0		
Meat/bone meal in GF diet			0.10	
Yes	21	66.7		
No	87	47.1		
Animal/Veg. fat in GF diet			0.18	
Yes	50	44.0		
No	68	56.9		
Yes				
No				
Yes				
No				
Buildings restrict birds			0.83	
None	15	53.3		
Some	58	53.4		
All	34	47.1		
Buildings restrict cats			0.66	
None	18	61.1		
Some	48	50.0		

All	41	48.8		

Factor	n	Percent positive	P value	OR
Buildings restrict rats/mice			0.19	
None	52	59.6		
Some	29	48.3		
All	26	38.5		
Buildings restrict dogs			0.98	
None	13	53.8		
Some	43	51.2		
All	51	51.0		
Feeding on wildlife carcasses			0.54	
Can be or is	28	46.4		
Definitely not	79	53.2		
Cattle on operation			0.88	
Yes	47	53.2		
No	58	51.7		
Sheep on operation			0.57	
Yes	5	40.0		
No	100	53.0		
Goats on operation			0.92	
Yes	4	50.0		
No	101	52.5		
Cats on operation			0.75	
Yes	75	53.3		
No	30	50.0		
Feral pigs in county			0.21	
Yes	10	70.0		
No	97	49.5		
Salmonella shedding in GF			0.20	
Yes	71	54.9		
No	38	42.1		
Region			0.04	
Non-central states	36	36.1		
Central states	77	57.1		

Development of final logistic regression model

A logistic regression model was constructed for each variable which passed the screening cut-off p value of <0.25 (highlighted above) controlling for region. Variables were placed in a full model if the Wald chi-square statistic had a p value < 0.25, with the exception of v540 which was dropped due to insufficient number of observations. The likelihood ratio testing the global null hypothesis for the full model was 0.006 indicating that some or all of the variables contributed to a model that was significantly better than a model with all coefficients = 0. A reduced model was generated based on the four variables which contributed most to the significance of the full model, i.e. those with a Wald chi-square value greater than 2 (and therefore a p value < 0.1).

Model	df	-2LogL	Difference	
Full	7	115.73		
Reduced	4	121.99	6.26 (3)	X2(3)=7.8

This table demonstrates that the reduced model is not significantly different than the full model since the estimated chi square with 3 degrees of freedom is less than the chi square with alpha=0.05. Therefore, the reduced model is better and is presented below as the final logistic regression model for estimating risk factors associated with sites positive for Yersinia.

Final Logistic Regression Model:

Factor	P value	Odds Ratio	95% CI
Region			
Central states			
Non-central states	0.013	0.3	0.1 - 0.8
Vaccination for E. coli			
No			
Yes	0.023	3.0	1.2 - 7.5
Percent deaths due to Scours			
0 %			
1-100%	0.022	3.5	1.2 - 10.5
Meat/bone meal in GF diet			
No			
Yes	0.017	4.1	1.3 - 13.3

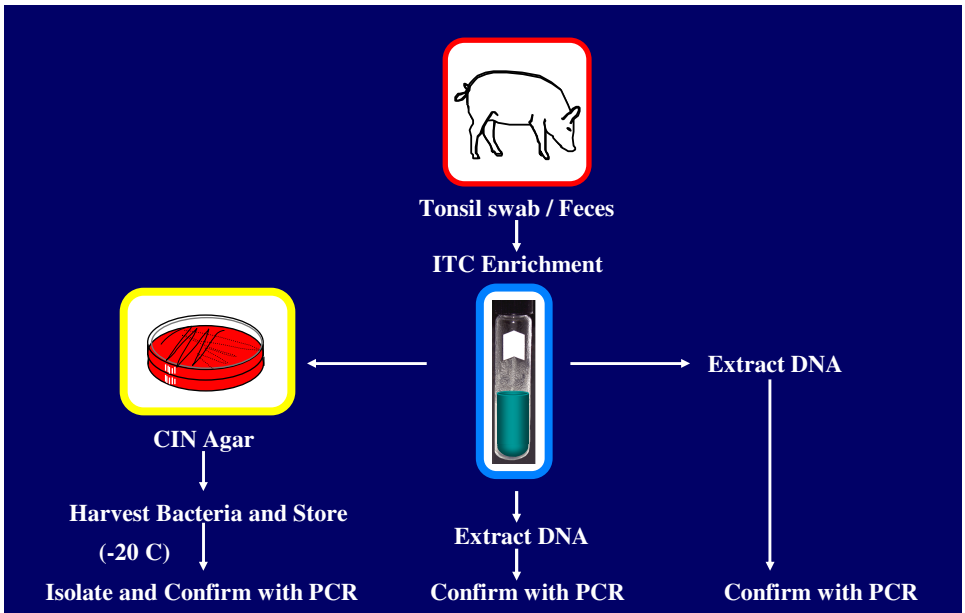

FIGURE 1. Flow chart for detection of *Y. enterocolitica* from feces and tonsil swab.

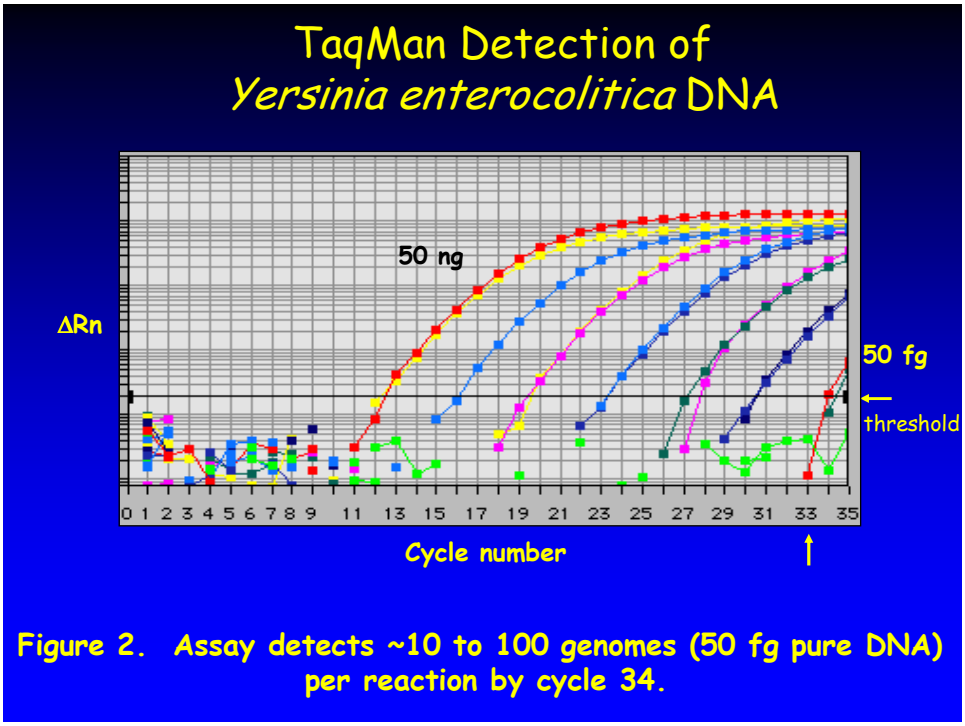

Figure 2. Assay detects ~10 to 100 genomes (50 fg pure DNA) per reaction by cycle 34.